Research Article

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Berries grown in Brazil: anthocyanin profiles and biological properties

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Abstract

BACKGROUND: Phytochemical profiles of two Brazilian native fruits – *pitanga* (red and purple) and *araçá* (yellow and red) – as well as strawberry cultivars Albion, Aromas and Camarosa, blackberry cultivar Tupy and blueberry cultivar Bluegen cultivated in Brazil were characterized for total phenolic content and total anthocyanin content by liquid chromatography coupled to a photodiode array and a quadrupole time-of-flight mass spectrometer. Radical scavenging, antiherpes and cytotoxic activities of these berry extracts were also evaluated.

RESULTS: Blueberry presented the highest total anthocyanin content (1202 mg cyanidin-*O*-glucoside equivalents kg^{-1} fresh fruit), while strawberry cultivar Aromas presented the highest total phenolic content (13 550 mg gallic acid equivalents kg^{-1} fresh fruit). Liquid chromatographic–mass spectrometric analysis resulted in the identification of 21 anthocyanins. To the best of our knowledge this is the first report of cyanidin-*O*-glucoside in yellow and red Araçá fruit and the first time eight anthocyanins have been reported in *pitanga* fruits. DPPH and ABTS assays showed that blueberry cultivar Bluegen, blackberry cultivar Tupy and *pitanga* (red and purple) showed the most promising antiradical activities, respectively. No relevant cytotoxicity against three cancer cell lines or antiherpes activity was detected under the experimental conditions tested.

CONCLUSION: Total anthocyanin content of all fruits had a strong positive correlation with their free radical scavenging activity, suggesting anthocyanins contribute to the antioxidant potential of these fruits.

Keywords: berries; anthocyanins; LC/PDA/Q-TOF-MS; *Eugenia uniflora*; *Psidium cattleianum*

INTRODUCTION

Berries constitute a large group of functional foods whose consumption has demonstrated a broad spectrum of positive effects in human health, including those in cardiovascular disorders, inflammatory conditions, metabolic syndrome, as well as distinct degenerative diseases. These effects have been related to specific phytochemicals, mainly phenolic compounds, such as phenolic acids, tannins and anthocyanins, the major compounds present in berries, which also improve neuronal and cognitive brain functions and protect genomic DNA integrity.[1,2]

Anthocyanins constitute the largest and likely the most important group of water-soluble plant pigments, and are responsible for the blue, purple and red color of diverse tissues. Anthocyanins have a flavilium cation as their core structure, with different sugars attached at position C3. Epidemiological data suggest a direct correlation between anthocyanin intake and low incidence of chronic and degenerative diseases, and anthocyanins also present a promising alternative to synthetic food dyes.[3,4]

Recently, the cultivation of certain berries in Brazil has expanded, especially in subtropical areas, and has been accompanied by an increase in consumption of blueberries (*Vaccinium virgatum* A.), strawberries (*Fragaria* × *ananassa* Duch.) and blackberries (*Rubus* sp.), as well as native Brazilian berries such as '*pitanga*' (*Eugenia uniflora* L.) and '*araçá*' (*Psidium cattleianum* S.).[5–8]

The investigation of anthocyanin composition of berries is relevant since it can be influenced by several factors such as geographical location, temperature and cultivation parameters.[9] Therefore, considering the limited number of investigations related to the chemical content of both Brazilian wild berries cited above, the present study aimed to characterize the anthocyanin profiles of different berries grown in Brazil. In addition, their antioxidant potential, antiherpes effects, as well as their cytotoxicity against human cancer cell lines, were evaluated.

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MATERIALS AND METHODS

Plant material

Fruit of red and purple varieties of *Eugenia uniflora* (*pitanga*), yellow and red varieties of *Psidium cattleianum* (*araçá*), *Rubus* sp. (blackberry) cultivar Tupy and *Vaccinium virgatum* (blueberry) cultivar Bluegen were cultivated at Empresa Brasileira de Pesquisa Agropecuária de Clima Temperado (EMBRAPA) in Pelotas, RS, Brazil (31° 40′ 50.6″ S, 52° 26′ 23.1″ W). *Fragaria* × *ananassa* (strawberry) cultivars Albion, Aromas and Camarosa were cultivated at the *Universidade de Passo Fundo* in Passo Fundo, RS, Brazil (28° 13′ 58.5″ S; 52°23′ 00.7″ W). After harvesting, fruits were stored at −20 °C until extraction and analyses.

Extraction procedures

Extracts of all fruits were prepared by sonication (Ultrasonic Cleaner 1450, Unique) using 1 g frozen fruits milled in an electric blender (LAR4, METVISA®, Brazil) and 60 mL methanol for 50 min at room temperature (23 ± 2 °C). The extracts were filtered in qualitative filter paper (particle retention: 4–12 μm) and concentrated to dryness under reduced pressure yielding the crude extracts. Each extract was then solubilized in distilled water (30 mL), freeze dried and then lyophilized. All extracts were stored at −20 °C for no longer than 60 days prior to phytochemical analyses and biological assays. All extractions were performed in triplicate.

Liquid chromatography–photodiode array detection–quadrupole time-of-flight–mass spectrometry (LC/PDA/Q-TOF-MS) of anthocyanins

Anthocyanins were individually identified and quantified by ultra-performance liquid chromatography (Acquity-UPLC™) coupled to a photodiode array detector (PDA) and a high-resolution mass spectrometer (Xevo® G2 QTof model, Waters®) equipped with an electrospray ionization source (ESI) operating in positive mode. The chromatographic separation was performed using a Synergi™ column (Phenomenex®, i.d. 4 μm, 150 × 2.0 mm) at 40 °C and the injection volume was set at 5 μL. The elution was performed using an aqueous phase consisting of formic acid 2% (solvent A) and acetonitrile containing 1% formic acid (solvent B). A linear gradient according to the following conditions was used: 0–10 min, 5–12% B; 10–29 min, 12–18% B; 29–33 min, 18% B; 33–34 min, 5% B with a constant flow of 0.4 mL min^{-1}. The detection was performed at 520 nm, and the range of spectral scanning in the visible region ranged from 450 to 600 nm (PDA). Mass scanning ranged from *m/z* 200 to 1500 with a scan time of 0.5 s. MS/MS analyses were performed using a collision energy ramp (10–30 eV) with argon as the collision gas. The capillary voltage of 1.0 kV, source block temperature of 120 °C, desolvation temperature of 600 °C, nebulizer nitrogen flow rate of 80 L h^{-1}, desolvation nitrogen gas flow of 800 L h^{-1} and cone voltage of 40 V were used, controlled by MassLynx v.4.1 software for data acquisition and processing. All samples were analyzed in triplicate; the methodology was validated, and specificity, linearity, accuracy, precision, and detection and quantitation limits were measured.

Total phenolic content (TPC)

TPC was determined by the Folin–Ciocalteu assay.[10] Samples and standards were analyzed in triplicate and the results were expressed as milligrams of gallic acid equivalents per gram of fresh fruit (mg GAE g^{-1} FF). A gallic acid standard curve was prepared with concentrations ranging from 60 to 300 μg mL^{-1} ($R^2 = 0.998$).

Total anthocyanin content (TAC)

The determination of total monomeric anthocyanin content was performed using the pH differential method[11] at two wavelengths (520 and 700 nm). The molar absorptivity coefficient (ε) used for cyanidin-3-*O*-glucoside in a methanol solution was 26 600 L mol^{-1} cm^{-1}. Results were expressed as cyanidin-3-*O*-glucoside equivalents in milligrams per 100 g of fresh fruit (mg CGE 100 g^{-1} FF).

Radical scavenging assays

DPPH assay

Free DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was assayed as previously described by Sánchez-Moreno *et al.*[12] Briefly, 100 mL of five different concentrations of each sample was added to 3.9 mL of a methanol solution of DPPH (60 μmol L^{-1}). After 60 min, absorbance was measured at 515 nm (Lambda 25 UV–visible, PerkinElmer®). Each sample was analyzed in triplicate, and the concentration of each extract that reduced 50% of DPPH concentration (EC_{50}) was expressed as mg mL^{-1}.

ABTS assay

Free ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was assayed using the method previously described.[13] Briefly, the ABTS radical was produced by mixing ABTS diammonium salt (7 mmol L^{-1}) and potassium persulfate (2.5 mmol L^{-1}). After 16 h, the radical produced was diluted to produce a solution with an absorbance of 0.7 at 734 nm. Finally, 10 μL of five different concentrations of each sample was added to 1 mL ABTS radical solution. After 5 min, absorbance was measured at 734 nm (Lambda 25 UV–visible, PerkinElmer®). Each sample was analyzed in triplicate, and the concentration of each extract that reduced 50% of ABTS concentration (EC_{50}) was expressed as mg mL^{-1}.

Cytotoxicity screening

Cytotoxic screening was conducted with three human cancer cell lines: A549 (non-small cell lung cancer cells, ATCC: CCL-185), RD (rhabdomyosarcoma, Instituto Adolfo Lutz, SP, Brazil) and DU145 (prostate carcinoma, DSMZ, ACC 261, Braunschweig, Germany). A549 and RD cells were cultivated in Eagle's minimum essential medium (MEM; Gibco, Carlsbad, CA, USA), and DU145 cells were grown in Dulbecco's modified Eagle's medium (DMEM; with high glucose and 1% L-glutamine, Gibco). All cell lines were supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained at 37 °C and 5% CO_2 in a humidified atmosphere. Cytotoxic screening was performed by the sulforhodamine B (SRB) assay.[14] Each cell line was exposed to different concentrations of the crude fruit extracts for 48 h. Paclitaxel (Sigma-Aldrich) was used as positive control. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced cell viability by 50% when compared to untreated controls, and was determined for each sample in triplicate.

Antiherpes screening

Two tests for determining anitherpes activity of fruit crude extracts were performed. First, the cytotoxicity of the extracts on Vero cells (ATCC: CCL81) was evaluated using the sulforhodamine B (SRB) assay as described above. These cells were grown in MEM supplemented with 10% FBS at 37 °C and 5% CO_2 for 24 h, and exposed to different concentrations of the extracts for 48 h. The

50% cytotoxic concentration (CC_{50}) of each sample was defined as the concentration that reduced Vero cell viability by 50% when compared to untreated controls. Second, the potential antiherpes (anti-HSV-1) activity was screened by plaque number reduction assay, as described previously.[15] Vero cells were infected with approximately 100 PFU of HSV-1 (KOS strain – acyclovir sensitive; Faculty of Pharmacy, University of Rennes I, Rennes, France) for 1 h at 37 °C. The fruit extracts were added at non-cytotoxic concentrations after viral infection. Then, cells were washed with PBS, overlaid with MEM containing 5% carboxymethylcellulose (Sigma-Aldrich) in the presence or absence of different concentrations of the fruit extracts and incubated for 48 h. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich) and viral plaques were counted using a stereo microscope. The concentration of each extract that inhibited viral replication by 50% (IC_{50}) when compared to untreated controls was determined. Acyclovir (ACV, Sigma-Aldrich) was used as positive control.

Data analysis

Statistical analyses were conducted using Graphpad Prism 6 software. An analysis of variance (ANOVA) was used to test differences among samples. Newman–Keuls multiple range test was used to verify differences. Pearson's correlation was used to determine whether there was a statistically significant relationship between two variables. Data are presented as means ± standard deviations.

RESULTS AND DISCUSSION

Anthocyanin identification

The identification of anthocyanins was initially established by the presence of a characteristic absorption band at wavelengths ranging from 500 to 550 nm in the visible region of the electromagnetic spectrum.[16] Identification of anthocyanins was based on tandem mass spectra and exact mass. Mass spectral data showed seven types of anthocyanins: those derived from pelargonidin (*m/z* 271), cyanidin (*m/z* 287), peonidin (*m/z* 301), delphinidin (*m/z* 303), petunidin (*m/z* 317), malvidin (*m/z* 331) and carboxypyranpelargonidin (*m/z* 339). The sugar moiety was determined by tandem mass fragmentation spectra and by the elution order of anthocyanins. As previously described, glucose and galactose were the predominant sugars attached to the identified anthocyanins.[17] To confirm identification, the exact mass and the spectra obtained were compared to those of MassBank® (https://www.massbank.jp) and Metlin® (https://metlin.scripps.edu) databases. Twenty-one anthocyanins were detected in the fruits herein investigated, of which eight were detected for the first time in the respective species: delphinidin-*O*-galactoside, cyanidin-*O*-galactoside, petunidin-*O*-galactoside, pelargonidin-3-*O*-glucoside, malvidin-*O*-galactoside, malvidin-*O*-pentoside and malvidin-*O*-acetylhexoside in *pitanga* fruits; and cyanidin-3-*O*-glucoside in *araçá* fruits.

Pitangueira is a native plant from Brazil that produces purple or red-colored fruit called *pitanga*. The LC/PDA/Q-TOF-MS of *pitanga* fruit showed 11 different anthocyanins (Table 1), and among these only cyanidin-*O*-glucoside (**5**),[18,19] delphinidin-*O*-glucoside (**2**)[19] and one derivative of malvidin[6] have been previously described for this fruit. Additionally, eight anthocyanins were identified in the red variety for the first time: delphinidin-*O*-galactoside (**1**), cyanidin-*O*-galactoside (**3**), pelargonidin-*O*-glucoside (**10**), petunidin-*O*-galactoside (**11**), pelargonidin-*O*-rutinoside (**13**), malvidin-*O*-galactoside (**15**), malvidin-*O*-pentoside (**20**) and malvidin-*O*-acetylhexoside (**21**). Four anthocyanins were identified in the purple *pitanga* variety and two of them – cyanidin-*O*-glucoside (**5**) and delphinidin-*O*-glucoside (**2**) – had previously been described.[18,19] The other two anthocyanins found – cyanidin-*O*-galactoside (**3**) and pelargonidin-*O*-glucoside (**10**) – were identified for first time in this variety.

In relation to *araçá* fruit, only one cyanidin derivative has been previously described.[20] In the current study, only cyanidin-*O*-glucoside (**5**) was identified in both *araçá* varieties. To the best of our knowledge, this compound is described here for the first time.

Another berry analyzed in this study was blackberry – a species more widely cultivated than *pitanga* or *araçá*. The anthocyanin profile of blackberries has been widely described and encompasses 12 anthocyanin derivatives of cyanidin, peonidin, pelargonidin and malvidin.[21] In the present study, four anthocyanins were identified in blackberry cultivar Tupy: cyanidin-*O*-galactoside (**3**), cyanidin-*O*-glucoside (**5**), cyanidin-*O*-rutinoside (**7**) and pelargonidin-*O*-glucoside (**10**), which is in agreement with previous reports for this fruit.[21]

Three strawberries cultivars (Camarosa, Aromas and Albion) were also investigated, and cyanidin-*O*-glycoside (**5**), pelargonidin-*O*-glucoside (**10**), pelargonidin-*O*-rutinoside (**13**) and carboxypyranopelargonidin-*O*-hexoside (**18**) were identified, which have been previously described in cultivars of *Fragaria* × *ananassa*.[22,23]

Although the production of blueberries is more restricted to the Northern Hemisphere, the berries evaluated here were cultivated in southern Brazil. As previously described,[24,25] blueberries presented the highest anthocyanin content in comparison to the other fruits examined here. As expected according to the LC/MS analyses, blueberries showed the highest anthocyanin complexity among all berry fruits herein investigated, with 15 anthocyanins identified: delphinidin-*O*-galactoside (**1**), delphinidin-*O*-glucoside (**2**), cyanidin-*O*-galactoside (**3**), delphinidin-*O*-pentoside (**4**), cyanidin-*O*-glucoside (**5**), petunidin-*O*-galactoside (**6**), petunidin-*O*-glucoside (**8**), cyanidin-*O*-pentoside (**9**), peonidin-*O*-galactoside (**11**), petunidin-*O*-pentoside (**12**), peonidin-*O*-glucoside (**14**), malvidin-*O*-galactoside (**15**), malvidin-*O*-glucoside (**16**), peonidin-*O*-pentoside (**19**) and malvidin-*O*-pentoside (**20**). All compounds have been previously described in blueberry.[24,25]

Finally, it is important to emphasize that among all anthocyanins identified, only cyanidin-*O*-glucoside (**5**) was present in all nine berry extracts analyzed.

Quantification of individual anthocyanins

All anthocyanins identified in fruit extracts were also quantified individually by UPLC-PDA-ESI-MS. The methodology was validated according to ICH guidelines (2005)[26] using cyanidin-3-*O*-glucoside as an analytical standard, and the parameters evaluated were accuracy, precision, specificity, linearity, and limits of detection and quantification (Table 2). Among the fruits analyzed it was possible to determine the presence of one major anthocyanin in each fruit berry, with the exception of blueberry cultivar Bluegen.

Cyanidin-*O*-glucoside represented 45% and 80% of TAC in red and purple *pitanga* varieties, respectively (Table 3). In blackberries, cyanidin-*O*-galactoside represented more than 90% of TAC. All strawberry cultivars presented pelargonidin-*O*-glucoside as the predominant anthocyanin with 85% of TAC. Blueberry fruit showed the highest complex mixture of anthocyanins and the

Table 1. Anthocyanins identified in nine berries evaluated

Anthocyanin	Retention time (min)	λ_{max} (nm)	Molecular formula	$[M]^+$ *m/z* (error, ppm)	Main fragments*m/z*	Fruit berries
(1) Delphinidin-*O*-galactoside	6.17	507	$C_{21}H_{21}O_{12}^+$	465.1060 (5.8)	303	Red and purple *pitanga*; blueberry
(2) Delphinidin-*O*-glucoside	6.69	507	$C_{21}H_{21}O_{12}^+$	465.1060 (5.8)	303	Red and purple *pitanga*; blueberry
(3) Cyanidin-*O*-galactoside	7.70	515	$C_{21}H_{21}O_{11}^+$	449.1065 (−4.2)	287	Red and purple *pitanga*; blackberry; Blueberry
(4) Delphinidin-*O*-pentoside	7.84	505	$C_{20}H_{19}O_{11}^+$	435.0892 (3.0)	303	Blueberry
(5) Cyanidin-*O*-glucoside	8.49	515	$C_{21}H_{21}O_{11}^+$	449.1065 (−4.2)	287	Red and purple *pitanga*; yellow and red *Araçá*; strawberry cv. Albion, Aromas and Camarosa; blackberry, blueberry
(6) Petunidin-*O*-galactoside	8.95	522	$C_{22}H_{23}O_{12}^+$	479.1161 (−6.1)	317	Blueberry, red *pitanga*
(7) Cyanidin-*O*-rutinoside	9.14	517	$C_{27}H_{31}O_{15}^+$	595.1705 (7.1)	449	Blackberry
(8) Petunidin-*O*-glucoside	9.59	522	$C_{22}H_{23}O_{12}^+$	479.1161 (1.1)	317	Blueberry
(9) Cyanidin-*O*-pentoside	9.64	517	$C_{20}H_{19}O_{10}^+$	419.0931 (−4.7)	287	Blueberry
(10) Pelargonidin-*O*-glucoside	10.29	500	$C_{21}H_{21}O_{10}^+$	433.1130 (1.2)	271	Red and purple *Pitanga*; strawberry cv. Albion, Aromas and Camarosa; blackberry
(11) Peonidin-*O*-galactoside	10.86	517	$C_{22}H_{23}O_{11}^+$	463.1215 (−5.4)	301	Blueberry
(12) Petunidin-*O*-pentoside	10.86	517	$C_{21}H_{21}O_{11}^+$	449.1087 (−0.1)	317	Blueberry
(13) Pelargonidin-*O*-rutinoside	11.09	500	$C_{27}H_{31}O_{14}^+$	579.1721 (1.2)	433; 271	Strawberry cv. Albion, Aromas and Camarosa
(14) Peonidin-*O*-glucoside	11.76	517	$C_{22}H_{23}O_{11}^+$	463.1215 (−5.4)	301	Blueberry
(15) Malvidin-*O*-galactoside	11.88	530	$C_{23}H_{25}O_{12}^+$	493.1326 (−4.1)	331	Red *Pitanga*; blueberry
(16) Malvidin-*O*-glucoside	12.69	530	$C_{23}H_{25}O_{12}^+$	493.1326 (−4.1)	331	Blueberry
(17) Cyanidin-*O*-malonylhexoside	12.87	518	$C_{24}H_{23}O_{14}^+$	535.1062 (−4.9)	449; 287	Blackberry
(18) Carboxypyranpelargonidin-*O*-hexoside	13,00	495	$C_{24}H_{21}O_{12}^+$	501.1014 (−3.8)	339	Strawberry cv. Albion, Aromas and Camarosa
(19) Peonidin-*O*-pentoside	13.03	515	$C_{21}H_{21}O_{10}^+$	433.1101 (−7.9)	301	Blueberry
(20) Malvidin-*O*-pentoside	14.22	522	$C_{22}H_{23}O_{11}^+$	463.1207 (-7.1)	331	Red *Pitanga*; blueberry
(21) Malvidin-*O*-acetylhexoside	20.29	528	$C_{25}H_{27}O_{13}^+$	535.1425 (-5.0)	331	Red *Pitanga*

Table 2. Parameters of the validated analytical methods for high-performance liquid chromatography detected by mass spectrometry for quantification of all anthocyanins expressed in cyanidin-3-*O*-glucoside

		Precision		Accuracy			
Compound	Concentration (μg mL^{-1})	Repeatability SDR(%)	Intermediate precision SDR(%)	Mean(%)	SDR(%)	LOD (μg mL^{-1})	Linearity (R^2)
Cyanidin-3-*O*-glucoside	0.04	5.5	5.0	105.0	4	0.01	
	3.12	3.5	3.8			0.02	0.998
	25.00	3.0	3.2				

SDR, standard deviation relative; LOQ, limit of quantification; LOD, limit of detection.

highest TAC, but they did not show one major anthocyanin compound.

Total phenolic content (TPC)

The TPC values of the evaluated berries are shown in Table 4. Among *pitanga* fruits, the purple variety showed a higher TPC (11 400 mg kg^{-1}) than the red one (8280 mg kg^{-1}). Denardin *et al.*[6] detected 7990 mg TPC kg^{-1} fresh fruit for purple *pitanga*, while Filippi *et al.*[27] detected 19 810 mg TPC kg^{-1} fresh fruit.

The strawberry cultivar Albion presented the lowest TPC when compared to the other cultivars evaluated. The TPC in all cultivars was higher than previously found in cultivar Camarosa (2600 mg GAE kg^{-1} fresh fruit),[28] in cultivar Aromas (2100 mg GAE kg^{-1} fresh fruit),[29] and in cultivar Albion (1700 mg GAE kg^{-1} fresh fruit).[30]

Table 3. Individual anthocyanin content of nine berries evaluated

Anthocyanin	Red *pitanga*	Purple *pitanga*	Yellow *araçá*	Red *araçá*	Strawberry cv. Camarosa	Strawberry cv. Aromas	Strawberry cv. Albion	Blackberry cv. Tupy	Blueberry cv. Bluegen
(1) Delphinidin-*O*-galactoside	3.2 ± 0.13	80.9 ± 3.48	nd	nd	nd	nd	nd	nd	265.2 ± 13.26
(2) Delphinidin-*O*-glucoside	1.1 ± 0.05	0.9 ± 0.04	nd	nd	nd	nd	nd	nd	22.9 ± 1.05
(3) Cyanidin-*O*-galactoside	9.1 ± 0.46	41.0 ± 2.05	nd	nd	nd	nd	nd	2.9 ± 0.12	49.6 ± 2.38
(4) Delphinidin-*O*-pentoside	nd	nd	nd	nd	nd	nd	nd	nd	113.6 ± 4.66
(5) Cyanidin-*O*-glucoside	69.1 ± 3.32	582.9 ± 22.73	0.4 ± 0.02	11.7 ± 0.60	2.3 ± 0.1	8.2 ± 0.38	3.8 ± 0.15	797.0 ± 33.47	2.9 ± 0.11
(6) Petunidin-*O*-galactoside	7.5 ± 0.31	nd	nd	nd	nd	nd	nd	nd	209.3 ± 10.47
(7) Cyanidin-*O*-rutinoside	nd	nd	nd	nd	nd	nd	nd	53.9 ± 2.75	nd
(8) Petunidin-*O*-glucoside	nd	nd	nd	nd	nd	nd	nd	nd	6.7 ± 0.31
(9) Cyanidin-*O*-pentoside	nd	nd	nd	nd	nd	nd	nd	nd	12.6 ± 0.54
(10) Pelargonidin-*O*-glucoside	1.1 ± 0.04	2.4 ± 0.10	nd	nd	150.8 ± 6.03	227.9 ± 11.40	222.5 ± 9.35	0.6 ± 0.02	nd
(11) Peonidin-*O*-galactoside	nd	nd	nd	nd	nd	nd	nd	nd	12.1 ± 0.48
(12) Petunidin-*O*-pentoside	nd	nd	nd	nd	nd	nd	nd	nd	67.9 ± 3.46
(13) Pelargonidin-*O*-rutinoside	nd	nd	nd	nd	19.2 ± 0.81	10.5 ± 0.41	13.8 ± 0.66	nd	nd
(14) Peonidin-*O*-glucoside	nd	nd	nd	nd	nd	nd	nd	nd	1.2 ± 0.06
(15) Malvidin-*O*-galactoside	37.2 ± 1.86	nd	nd	nd	nd	nd	nd	nd	333.3 ± 13.00
(16) Malvidin-*O*-glucoside	nd	nd	nd	nd	nd	nd	nd	nd	7.6 ± 0.31
(17) Cyanidin-*O*-malonylhexoside	nd	nd	nd	nd	nd	nd	nd	18.0 ± 0,70	nd
(18) Carboxypyranpelargonidin-*O*-hexoside	nd	nd	nd	nd	1.1 ± 0.05	0.8 ± 0.03	0.5 ± 0.02	nd	nd
(19) Peonidin-*O*-pentoside	nd	nd	nd	nd	nd	nd	nd	nd	2.6 ± 0.10
(20) Malvidin-*O*-pentoside	16.3 ± 0.75	nd	nd	nd	nd	nd	nd	nd	94.3 ± 4.34
(21) Malvidin-*O*-acetylhexoside	3.3 ± 0.14	nd	nd	nd	nd	nd	nd	nd	nd

Results are expressed as mg kg^{-1} ± standard deviation of fresh fruits. nd, not detected.

Table 4. Total phenolic (TPC) and total anthocyanin (TAC) content of nine berries evaluated

	TPC ± SD	TAC ± SD
Red *pitanga*	8280 ± 140a	14.8 ± 2.2a
Purple *pitanga*	11400 ± 120b	708.1 ± 6.6b
Yellow *araçá*	3820 ± 120c	0.4 ± 0.1c
Red *araçá*	7190 ± 150d	1.7 ± 1.8d
Strawberry cv. Camarosa	11660 ± 200b	173.5 ± 9.8e
Strawberry cv. Aromas	13550 ± 230e	247.5 ± 3.1f
Strawberry cv. Albion	10810 ± 250f	240.7 ± 3.2f
Blackberry cv. Tupy	9210 ± 60 g	872.4 ± 2.9 g
Blueberry cv. Bluegen	10900 ± 250f	1202.0 ± 7.6 h

Data are expressed as mg kg^{-1} fresh fruit ± standard deviation, and represent the mean of three independent experiments. Different letters within each column indicate significant differences (Newman–Keuls, $P \leq 0.05$).

Table 5. EC_{50} values (mg mL^{-1}) of berries obtained from DPPH and ABTS radical scavenging assays

Berry	DPPH ± SD	ABTS ± SD
Red *pitanga*	52.02 ± 0.47a	125.70 ± 3.85a
Purple *pitanga*	17.76 ± 0.27b	59.38 ± 2.09b
Yellow *araçá*	49.26 ± 0.79c	91.94 ± 1.02c
Red *araçá*	60.11 ± 2.36d	141.00 ± 2.80e
Strawberry cv. Camarosa	60.45 ± 1.08d	95.96 ± 5.99d
Strawberry cv. Aromas	65.06 ± 0.30e	114.10 ± 2.72d
Strawberry cv. Albion	63.60 ± 0.86e	125.97 ± 1.37a
Blackberry cv. Tupy	16.84 ± 0.50b	58.84 ± 1.20b
Blueberry cv. Bluegen	9.82 ± 0.48f	49.97 ± 5.11f
Gallic acid	0.03 ± 0.005 g	0.03 ± 0.004 g

Data represent the mean of three independent experiments ± standard deviation. Different letters within each column indicate significant differences (Newman–Keuls, $P \leq 0.05$).

The TPC values of *araçá* fruit were 7190 (red) and 3820 (yellow) mg kg^{-1} fresh fruit. These results were slightly higher than those obtained by other authors, who detected TPC values ranging from 5370 to 7680 mg kg^{-1} fresh fruit.[6,31,32] Furthermore, blackberry and blueberry fruits showed TPC values of 9210 and 10 900 mg kg^{-1} fresh fruit, respectively. In this study, purple *Pitanga*, all three cultivars of strawberries, and blueberries were the species with the highest TPC values. Although phenolic compounds in berries have been shown to present relevant antioxidant capacity by several mechanisms such as scavenging free radicals, chelating transition metals, inhibiting pro-oxidant and oxidant enzymes, and preventing lipid oxidation, they are not related to biological activities investigated herein.

Total anthocyanin content (TAC)

Purple *pitanga* fruit showed a higher TAC when compared to the red variety. Similarly, red *araçá* fruits presented a higher TAC than the yellow variety. Among the berries evaluated, blueberries (1200 mg kg^{-1}), blackberries (870 mg kg^{-1}) and purple *pitanga* (710 mg kg^{-1}) showed the highest TAC values (Table 4).

Anthocyanin contents of strawberry fruit cultivars Camarosa and Aromas were 170 and 250 mg kg^{-1} of fresh fruit, respectively, which are slightly lower than those obtained by other researchers. For instance, previous studies reported TACs of 260 and 300 mg kg^{-1} of fresh fruit, respectively, in cultivar Camarosa.[35,36] Similar results were shown for cultivar Aromas, with a reported TAC of 2157 mg kg^{-1} fresh fruit.[31] Many variables can explain the differences found among studies, including the distinct molar extinction coefficient values (ε) used, which can range from 15 600 to 31 620.[16] In this work, $\varepsilon = 2600$ was used, which was calculated from our experimental data.

The completely conjugated structure of anthocyanins that allows electron delocalization results in very stable radical products, which is favorable when considering their antioxidant potential. Moreover, the degree and position of hydroxylation and methoxylation in the B ring affect their stability and reactivity, and thereby also their antioxidant action. The results herein obtained using the DPPH assay are in accordance with previous observations on the effects of hydroxylation and methoxylation in the B ring on the radical scavenging ability of anthocyanins. As previously described, the anthocyanidins lacking the *O*-diphenyl structure in the B ring (malvidin, pelargonidin, petunidin and peonidin) had lower scavenging efficiency toward the DPPH radical compared to cyanidin and delphinidin.[35]

Radical scavenging assays

Berries have been shown to possess important antioxidant activity.[6,36,37] In this work, radical scavenging activity was measured by two standard assays (DPPH and ABTS). The results showed statistically significant differences among the fruit extracts analyzed concerning their antioxidant potential, which can be associated with their anthocyanin contents. The highest activity in both assays was found for blueberry fruit (Table 5). On the other hand, strawberries showed the lowest antioxidant potential among the fruits examined, with an $EC_{50\,DPPH}$ value greater than 60 mg mL^{-1} for all cultivars.

According to data shown in Table 5, it can be noted that the radical scavenging potential of the samples was higher when obtained by the DPPH assay than by ABTS assay. However, the results obtained by different assays are not directly comparable due to the difference in the mechanism of radical capture occurring in each assay.[12,13] Pearson correlation analyses for both assays and TAC showed a strong correlation ($r_{DPPH} = 0.86$ and $r_{ABTS} = 0.82$) (Fig. 1), while TPC presented low negative correlations with the antioxidant potential of the samples ($r_{DPPH} = -0.04$ and $r_{ABTS} = -0.17$).

Many studies have reported the relevant radical scavenging potential of berries. In one of these studies, Huang *et al.*[37] showed high antioxidant activity for strawberry, blackberry and blueberry fruit using the DPPH and ABTS assays. Similar results were found for blackberry, *araçá* and *pitanga* fruit by the DPPH assay.[6] Arend *et al.*,[36] however, reported a strong correlation ($r = 0.97$) between TAC of strawberry concentrates and their radical scavenging potential by DPPH assay.[32]

Cytotoxicity against human cancer cell lines

All dried berry methanolic extracts, when evaluated at the maximum concentration of 0.5 mg mL^{-1}, were unable to reduce cell viability by 50% (CC_{50}) in all tested cancer cell lines. These results corroborate those obtained by Elisia *et al.*,[38] who showed no cytotoxic effects for blackberry extracts (0.1–10 mg mL^{-1}) against human cancer cell lines LNCaP, MCF7, and MDA-MB-453.[38] In addition, Kulisic-Bilusic et al.[39] also reported no cytotoxic effects for strawberry extracts (0.5–2 mg mL^{-1}) against HT-29 cells.[39] On

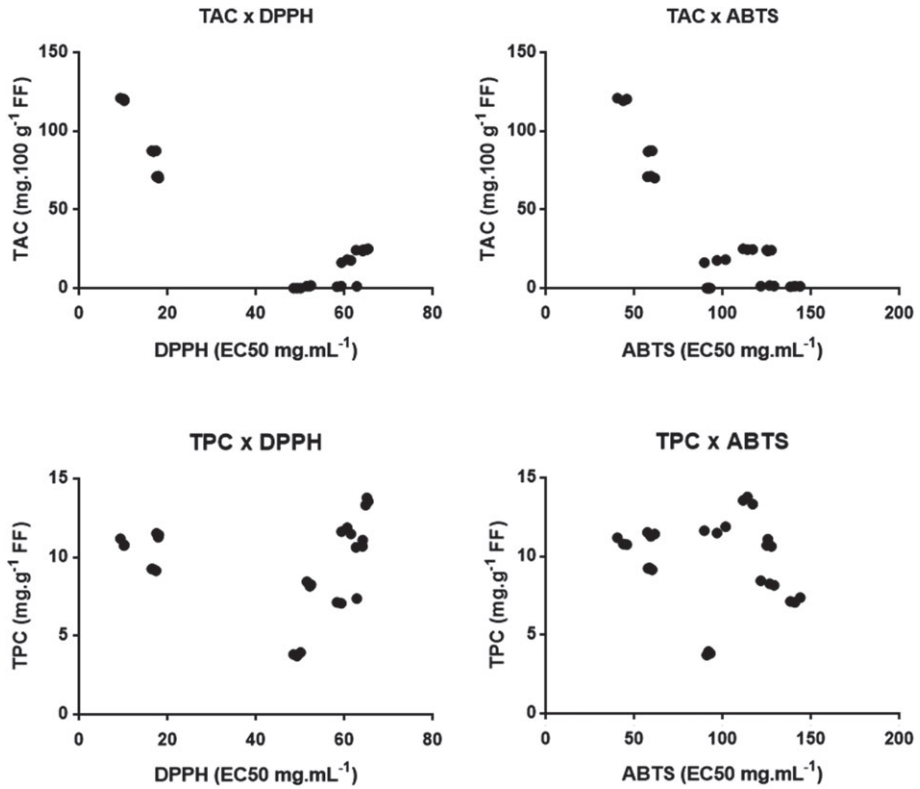

Figure 1. Pearson's correlation between TAC and TPC with DPPH and ABTS for nine berry extracts.

the other hand, Weaver *et al.*[40] showed cytotoxicity against cancer and normal human breast and prostate cell lines for a strawberry extract enriched in anthocyanins, and the maximum concentration tested was 10 μg mL^{-1}, indicating that they were not selective.

Antiherpes screening

The evaluation of antiherpes activity (anti-HSV-1, KOS strain) was performed using the same concentration of methanolic dried berry extracts described previously. Similarly, no inhibition of viral replication by 50% (IC_{50}) was observed for all tested samples. According to Cos *et al.*,[41] a concentration of 0.1 mg mL^{-1} should be the maximum concentration for plant extracts to obtain satisfactory antiviral activity. Therefore, the absence of inhibition of HSV-1 replication at 0.5 mg mL^{-1} indicates that the samples tested were not active under the experimental conditions of this study. While in a previous study inhibition of HSV-1 replication was observed with extracts of strawberry fruits,[42] all samples tested in the present study were not active.

CONCLUSION

Anthocyanin profiles of several berries cultivated in southern Brazil varied according to species. Blueberries showed the highest total anthocyanin content and the highest complexity of anthocyanic compounds. For *pitanga* fruit, the presence of eight anthocyanins not previously described in this species was detected. *Araçá* fruit showed the lowest total anthocyanin content compared to the other berries tested, and cyanidin-*O*-glucoside was identified for the first time in this fruit. The total anthocyanin content of all tested fruits showed a strong positive correlation with free radical scavenging activity but not with total phenolic content, suggesting anthocyanins as the major contributor to the antioxidant potential of these berries. Finally, the *in vitro* evaluation of cytotoxicity against human cancer cell lines and antiherpes activity did not present promising results under the experimental conditions tested.

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